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**Auditory sensitivity, spatial dynamics and amplitude of courtship
song in *Drosophila melanogaster***

Running title: Spatial dynamics of *Drosophila* courtship song

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ABSTRACT

Acoustic communication is an important component of courtship in *Drosophila melanogaster*. It takes the form of courtship song produced by males through the unilateral extension and vibration of a wing. Following the paradigm of sender-receiver matching, song content is assumed to match tuning in the auditory system, however, *D. melanogaster* audition is nonlinear and tuning dependent upon signal amplitude. At low stimulus amplitudes or in the absence of sound the antenna is tuned into song frequency, but as amplitude increases the antenna's resonance is shifted up by hundreds of Hertz. Accurate measurements of song amplitude have been elusive because of the strong dependency of amplitude upon the spatial geometry between sender and receiver. Here, we quantify *D. melanogaster* auditory directional sensitivity and the geometric position between the courting flies. We show that singing occurs primarily from positions resulting in direct stimulation of the female antenna. Using this information, we establish that the majority of song is louder than theoretically predicted and at these sound levels the female antenna should not amplify or tune into song. Our study implies that *Drosophila* hearing, and in particular its active mechanisms, could function in a broader context than previously surmised.

I. INTRODUCTION

Courtship behaviour in *Drosophila* culminates in a song that can determine mating success (Coen et al., 2014; von Schilcher, 1976a). Song is produced by the male vibrating an outstretched wing. Male and female rarely remain stationary, with the male 'dancing' around the female in order to find the best position from which to gain her attention. The male's song comprises 2 components: a sine component (a tone of ca. 160 Hz) and a pulse component (2-3 transient sinusoid cycles with a higher frequency content than sine song at ca. 200 Hz). These are produced in alternation with one another with pulses appearing at regular inter-pulse-intervals (IPI). The IPI is species specific and in *Drosophila melanogaster* lasts ca. 34 ms (Bennet-Clark and Ewing, 1967; Shorey, 1962). The female detects the male's song using her antennal ears, which are sensitive to the inherently directional, and rapidly attenuating velocity component of the sound wave (Bennet-Clark, 1971). The mechanics of *Drosophila* antennae, however, are nonlinear, whereby antennal frequency tuning (its resonance or best frequency; the frequency where a minimum amount of sound energy causes a large antennal displacement) is dependent upon sound amplitude (Albert et al., 2007; Albert and Kozlov, 2016; Göpfert and Robert, 2001, 2002; Kamikouchi et al., 2010; Riabinina et al., 2011). In silence or very low amplitude conditions, female antennal tuning matches the frequency content of the male song. However, as amplitude increases antennal spectral tuning shifts up by hundreds of Hertz, effectively detuning itself from song frequency. With amplitude dependent spectral tuning, the *Drosophila* auditory nonlinearity also amplifies quiet, low frequency, sounds. The adaptations in amplification and tuning of the *Drosophila* ear are actively generated by dynein motors within the mechanosensory neurons in the antennal pedicel (Karak et al., 2015; Nadrowski et al., 2008). Several mutant strains of *Drosophila* have been bred that have non-functional motors systems (Göpfert and Robert, 2003) which are extremely useful in understanding the role and mechanisms of active auditory mechanics.

The mobility of courting *Drosophila* has been anecdotally reported (von Schilcher, 1976b), and more recently examined in courting pairs (Coen et al., 2014, 2016), however, positional information

describing angles and distance between the courting male and female and how these parameters impact the absolute amplitude of sound impinging on the female antenna are lacking. The use of space by the communicating fly pair will determine the amplitude at the female's antennal receiver which, as a nonlinear system, critically determines its tuning and sensitivity (the transfer function between energy input and antennal mechanical response), and hence sender-receiver matching. However, even assuming static positional relationships between flies, little is known about actual song amplitude at both source (male wing) and receiver (female antenna). Early work offered theoretical estimates of song amplitudes between 80 to 95 dB sound velocity level (SVL re $5 \times 10^{-8} \text{ m s}^{-1}$) close to males (Bennet-Clark, 1971). To determine whether the antenna is tuned into song, it is critical to know song amplitude at the antenna and its sensitivity to stimuli from different angles of incidence. To acquire this information the distance between male and female during song production must be known, as well as their relative orientation and the directional and active nonlinear sensitivity of the female ear. Together, these factors determine sensory capability and information transfer during hearing. Establishing what females can hear during courtship enhances our understanding of mating success but also our overall appreciation of the sensory ecology of *D. melanogaster*.

Our aim is to characterise the position of song production and the directional sensitivity of the female antennae to sound stimuli. We also aim to provide the first empirical measurements of courtship song amplitude and discuss whether, at these sound levels, the female antenna is mechanically tuned into male song.

We examined the use of space in *D. melanogaster* courtship using frame-by-frame video analysis in conjunction with multi-microphone sound recordings in pairs of flies. The combination of these data with both mechanical directional response and nonlinearity of the female auditory system, song spectra and amplitude, reveals that acoustic signalling during courtship is louder than predicted, and for the majority of courtship attempts, leaves the antenna detuned from song frequencies. Further implications of these findings in a bioacoustics and ecological context are discussed.

II. MATERIALS AND METHODS

A. Fly strains and maintenance

Drosophila melanogaster Oregon R (No. 4269; Bloomington *Drosophila* Stock Centre, Bloomington, IN, USA), *btv*^{SP1} and 40AG13 (*FRT*^{40A} *FRT*^{G13}) were maintained on instant fly medium (Carolina Biological Supply Co., Burlington, NC, USA) at 25°C on a 12:12 hour light:dark cycle. Flies used in behavioural assays were isolated as imagos immediately after eclosion, sexed and inspected for wing and antennal damage.

B. Antennal vibration measurements

Measurement of antennal vibration using laser Doppler vibrometry (LDV) has been described in detail previously (Morley et al., 2012). Either a pseudorandom chirp or pure tone sine stimulus was generated using LDV PSV 8.6 software (Polytec GmbH, Waldbronn, Germany) and data acquired (National Instruments PCI-4451; Austin, TX, USA), amplified (Sony TAFE570; Tokyo, Japan) and played through a loudspeaker (Visaton FR 108 OHM; Visaton GmbH & Co, Haan, Germany). Three playback amplitudes were used 88 dB_{SVL} (1.25 mm s⁻¹), 68 dB_{SVL} (0.125 mm s⁻¹) and 48 dB_{SVL} (0.0125 mm s⁻¹) utilising a bench-top attenuator (JFW 50BR-009; JFW Industries Inc. IN, USA). The loudspeaker was fixed to the edge of a turntable and the fly positioned centrally, 4.5 cm from the centre of the loudspeaker. Stimuli were monitored using a pressure gradient microphone (Knowles NR-23158-000; Knowles, Itasca, IL, USA) via an integrating amplifier (modified after (Bennet-Clark, 1984)) fixed 5 mm vertically below the fly. Resonant frequency was extracted by fitting a simple harmonic oscillator using MATLAB (v.2011a; MathWorks, Inc., Natick, MA, USA). The three fly strains were used for the mechanical measurements: Oregon R wild type, *btv*^{SP1} and 40AG13 (*FRT*^{40A} *FRT*^{G13}) wild type. Both Oregon R and 40AG13 (*FRT*^{40A} *FRT*^{G13}) are wild type strains; the 40AG13 (*FRT*^{40A} *FRT*^{G13}) is the wild type genetic background that the mutant *btv*^{SP1} was generated from. The inclusion of 40AG13 (*FRT*^{40A}

FRT^{G13}) is therefore a necessary control. The *btv^{5P1}* mutant strain was used to investigate the involvement of active auditory mechanics in each experiment. This mutant strain has a linear auditory response with no measurable active auditory mechanics (Göpfert and Robert, 2003).

C. Behavioural assays

Courtship was recorded for pairs of flies three days post-eclosion. Socially naïve flies were introduced into a custom built, circular arena comprising a petri-dish bottom (Ø 25 mm) lined with filter paper, a cover slip top (Ø 24 mm; all Fisher Scientific UK Ltd., Loughborough, UK) and walls made of acoustically transparent nylon mesh (10 mm high), alleviating problems of internal acoustic reflections. Four calibrated pressure gradient microphones (with a flat frequency response up to 3 kHz; NR-23158, Knowles Electronics, Itasca, IL, USA) arranged accurately around and outside the arena in a fixed and known square geometry. Microphones were each connected to an integrating amplifier (modified after (Bennet-Clark, 1984)) and digitised via a USB soundcard (Maya44 USB; ESI Audiotechnik GmbH, Leonberg, Germany). Microphone output was recorded with Audacity (v. 1.3.6; The Audacity Team; <http://audacity.sourceforge.net>) and saved as 22.05 kHz, 16 bit mono .wav files. This behavioural arena thus allowed for free-field acoustic recordings of singing male *Drosophila*.

For courtship assays, a male fly was placed in the arena at least 10 minutes before introducing a female. Sound and video recordings commenced with the females' insertion into the arena. To monitor both male and female positions, courtship was filmed from above (Canon Mini DV Camcorder MVX460; Canon Inc., Tokyo, Japan) at 25 fps. Sound recordings were synchronised with video recordings via a loud tone that appeared on both the sound recordings and the video camera's audio track. Audio and video tracks were subsequently aligned to this signal using Adobe Premiere Pro CS5 (Adobe Systems, San Jose, California, USA). Only courtship sequences taking place on the floor (thus excluding side walls and ceiling) of the arena were considered for analysis. Experiments were carried out on a vibration isolation table (TMC 784-443-12R; Technical Manufacturing Corp., Peabody, MA, USA) in an anechoic chamber at $25^{\circ} \pm 1^{\circ}$ C. Assays were deemed successful when copulation occurred

within 15 minutes of the pair's first encounter. Non-copulating pairs were excluded from this study. X/Y positions, orientations, minor and major axis lengths were individually tracked using Ctrax (v. 0.1.5.2, BehavioralMicroarray Toolbox, v. 0.1.04; <http://ctrax.sourceforge.net/>; (Branson et al., 2009)). Sound recordings were bandpass filtered (50-1000 Hz, 4096 points FFT size, Hanning window) using Cool Edit Pro (v. 2.00; Syntrillium Software Corporation, Phoenix, AZ, USA). Song bouts were analysed with Avisoft-SASLab Pro (v. 5.1; Avisoft Bioacoustics, Berlin, Germany). Amplitudes of both pulse and sine song at the female antenna were calculated by triangulation, using the theoretical attenuation rate for particle velocity in the near field emitted by an acoustic dipole $A \propto r^{-3}$, where A is particle velocity amplitude and r the distance to the sound radiator (Bennet-Clark, 1971; Jackson and Robert, 2006). Sound and video files were manually synchronised and spatial position analysis and sound amplitudes were obtained using custom-written MATLAB scripts.

III. RESULTS

A. Spatial analysis in courtship

Courting pairs of flies were filmed and simultaneously recorded while free to move about a mesh-walled arena, offering uncluttered acoustical conditions and ample space for behavioural complexity. Videos were analysed frame-by-frame to determine the position of both male and female flies throughout courtship and song production. Spectro-temporal characteristics of sine and pulse song of 23 males (with 4338 individual pulses and 206 sine songs) were similar to those previously reported (Cowling and Burnet, 1981; von Schilcher, 1976a, 1976b; Wheeler et al., 1988); pulse frequency: 203.9 ± 17.1 Hz; interpulse interval: 38.7 ± 3.1 ms; sine frequency: 167.3 ± 19.7 Hz. Our analysis reveals that the production of courtship song is spatially dynamic; both sexes are highly mobile with males singing from a wide range of positions around the courted female (Fig. 1A, B; supplementary video 1&2 [1]). The majority of song (90%) is observed while the male is within 5.4 mm (ca. 2 body lengths) of the

female (Fig. 1C and Fig. 2A). Although males can sing from varying locations, in time, they converge predominantly at angular positions behind the courted female (Fig. 1A-C and Fig. 2B). To evaluate more accurately the geometry of acoustic emissions by males, the position of the acoustic radiator needs to be known. We estimated the centre of the acoustic radiator to be mid-way along the vibrating wing, the position of average vibration amplitude. As a male moved around the female, he switched from one wing to the other such that song transmission followed an unimpeded path to the female receiver. Thus, a male positioned behind and to the right of a female used his right wing and *vice versa* (as observed in 84% of 69 randomly selected singing events examined in 9 males). Taking the middle of the acoustic radiator as the location of sound production, the majority of song was produced from two symmetrical hotspots offset by ca. 40° from the female's longitudinal axis (Fig. 1C; right centre 140°, 27° width; left centre 226°, 42° width, Fig. 2B). It is from these two positions that males spend most time producing courtship songs (Fig. 1C; Fig. 2B).

B. Directional hearing and courtship geometry

As male orientation changes during courtship, the angle of incidence of sound on the female antenna is likely to vary. The mechanical response of the female antennal sound receiver was thus measured, using LDV, in response to analytical stimuli from different angles of incidence and three different stimulus amplitudes. The female antennae are most sensitive to incident stimuli along 2 axes; one running 45°-225° (with reference to the longitudinal axis, starting at the head, going clockwise) producing peak mechanical responses in the left antenna, and the other axis running 135°-315° for the right antenna (Fig. 3A). These axes of maximal antennal stimulation match with the angles of acoustic centres of song production during courtship (Fig. 3B, C; Fig. 1C). When the stimulus comes from anterior positions 45° either side of the longitudinal axis, a diminished mechanical response is produced in the ipsilateral receiver. Therefore, contrary to conventional logic of sound reception in tympanal hearing systems (Hoy and Robert, 1996), when a male sings in front of a female, the antenna contralateral to the source of sound receives a greater stimulus magnitude.

The nonlinearity of *D. melanogaster*'s auditory system dictates that both mechanical sensitivity and tuning depend on stimulus amplitude (Göpfert and Robert, 2002). Because the angle of incident sound determines stimulus amplitude, it is expected that the direction of the sound source will affect both tuning and mechanical sensitivity of the auditory receivers. At low stimulus amplitudes, the mechanical tuning difference between left and right antenna was measured to be over 150 Hz, indeed depending upon the angle of the incoming stimulus (Fig. 3D-F). At high stimulus amplitudes, and in the auditory mutant *btv*^{SP1} where only a passive state is possible, this response is diminished (to around 100 Hz at 88 dBSVL) or vanishes (*btv*^{SP1}) (Fig. 3D-F). The auditory mutant *btv*^{SP1} was used as a control to assess whether physical acoustic conditions could explain the observed angle-dependent mechanical responses. Altogether, this latter evidence demonstrates that active mechanics could influence directional detection.

C. Song amplitude influence on auditory tuning

Active antennal mechanics are deemed to tune *D. melanogaster* into courtship song. However, the receiver's frequency of best sensitivity and song frequency match only at low song amplitudes (<46 dBSVL) (Riabinina et al., 2011). To evaluate whether active auditory mechanics are employed by the female auditory system during the majority of courtship requires empirical knowledge of both stimulus amplitude and incident angle. We recorded song in courting pairs of *D. melanogaster* and calculated amplitudes of both pulse and sine song at the female antenna by triangulation.

Owing to known attenuation and exact courtship geometry, pulse song and sine song emitted by 23 males (from wing acoustic centres) is revealed here to expose the receiving female antennae to high amplitudes; pulse song: 99.2 dBSVL median, 22.4 dBSVL interquartile range; sine song: median 87.7 dBSVL, 25.0 dBSVL interquartile range (Fig. 4A). At these amplitudes the antenna is driven to its passive regime where it is tuned to frequencies around 700 Hz (Fig. 4A, B). This tuning is in fact hundreds of hertz above song frequencies, and results from a mechanical regime notably characterised by the absence of amplification.

IV. DISCUSSION

Males predominantly produce songs positioned behind and close to the courted female. The preferred singing locations are situated either side of the female midline, corresponding to the angles of best acoustic stimulation of the female antenna. We quantify song amplitude at the position of the female antenna, which is higher than predicted (Bennet-Clark, 1971), delivering particle velocity levels reported to induce hearing impairment when presented chronically (Christie et al., 2013). Although males have recently been found capable of dynamically modulating song amplitude with increasing distance between the pair (Coen et al., 2016), they appear to be maximising amplitude at the female antenna rather than ‘whispering’ and males producing abnormally quiet sine songs incur lower mating rates (Shirangi et al., 2013).

Our measurements of song amplitude provide the first empirical quantification of the particle velocity at the female antenna. We acknowledge that there are additional factors that could have some influence on song amplitude at the antenna, which are technologically difficult to capture in live courting flies. For example, viscous forces dominate the oscillatory flow around the head of *D. melanogaster* (Morley et al., 2012). The boundary conditions generated in these viscous regimes mean that the magnitude of particle velocity at the position of the antennae can be increased by around 5 dB, or likewise decreased depending on the incident angle of the stimulus (Morley et al., 2012). Additionally, the exact position of the female antenna is also within muscular control and may vary (ca. 15°) while she is moving during courtship (Mamiya et al., 2011). This positional uncertainty can in theory have an impact on the absolute velocity values, yet are unlikely to affect the median values we report that are 2-3 orders of magnitude above expected values for tuned antennae.

Remarkably, antennal tuning only matches the frequency of courtship song when stimulus amplitudes fall below 0.01 mm s⁻¹ (46 dB_{SVL}; Fig. 4). In effect, high amplitude courtship songs detune the receiver, making it less sensitive to low frequencies, a form of compressive protection. As demonstrated here,

such tuning is only rarely taking place when the male actively courts a female from their preferred positions, where amplitudes are regularly above 0.79 mm s⁻¹ (84 dBSVL). Morley et al. (2012) demonstrate that there can be an interantennal velocity difference of 25 dB, which leads to a reduced stimulation of the ipsilateral antenna. However, for the majority of sound signals produced during courtship, this reduction in stimulus amplitude, while changing antennal tuning by around 100Hz, would not lead to a substantial activation of the nonlinear amplifier (Fig. 4a, b), tuning it into frequencies of between 600-800Hz (song is 100-250Hz). With the song amplitudes measured here, only singing from far away, or singing from positions off-axis to the female's best sensitivity would require amplification and active auditory tuning in the detection of courtship song. We show that, due to its amplitude-dependent tuning, a pair of antennae on a courted female can be differentially tuned depending on the incident angle and amplitude of the sound stimulus (Fig. 3D-F), these differences being greatest when the sound is quiet or far away. Active mechanics can therefore play a role in auditory directional sensitivity, where the differential tuning of the antennae could help determine the position of the sound stimulus. From a receptor mechanics and sensitivity perspective, being sensitive to particle velocity in the acoustic near-field does not prevent the detection of sounds generated by distant yet sufficiently powerful sources. Therefore, the female antennae may also be tuned into male song when he is singing from a distance, perhaps while courting another fly.

Because males usually direct their acoustic signals to the female at close range, (90% of songs produced within 5.4 mm distance to female), courtship signals are deemed to be the dominant, if not the only signals (but see Jonsson et al., 2011; Versteven et al., 2017), relevant in the acoustic ecology of *D. melanogaster*. Surprisingly however, very little is known about the natural history of *D. melanogaster* (Reaume and Sokolowski, 2006), or the environment in which their hearing capabilities evolved. In most other insects, hearing is believed to have initially evolved to allow detection of predators and parasitoids (Strauß and Stumpner, 2015), with many silent insects that are unable to call having the ability to hear (for example Fournier et al., 2013; Lehmann et al., 2010; Lucas et al., 2014). Active auditory mechanics in *Drosophila*, in addition to augmenting the detection of courtship

song from a distance or from suboptimal angles, may also serve to extend hearing beyond directed intraspecific communication (courtship, aggression) and enhance other acoustic signals from sources further afield. Better characterisation of the natural acoustic environment and sensory ecology of *Drosophila* can provide a broader and more complete picture of the adaptive function of their active auditory mechanics. It is therefore reasonable to suggest that listening to sounds further afield, such as those of predators and parasitoids, could be another and important function of the non-linear *Drosophila* antenna.

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ENDNOTE

[1] See supplementary material at [**URL will be inserted by AIP**] for Supplementary videos 1 and 2.

Graphical depiction of pulse song bouts produced during two separate courtships showing the same fly pairs as Fig. 1 A and B, respectively. The blue triangle represents the male moving in the coordinate system of the female (both to scale), the tip being posterior, and the base anterior. Each bout is marked as a line and points mark the location of the production of each song pulse. Bouts are sequential, from first to last, with intervals between bouts omitted.

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FIGURE LEGENDS

Figure 1. (Color online) Spatial representation of male *D. melanogaster* acoustic centres (outstretched vibrating wings) around the female during pulse song production. A, B) Acoustic centre position for two different males. Points mark acoustic centres per pulse during song bouts, colors (online) represent individual bouts. Individual female fly outlines are true to scale (A: 16 songs, 181 pulses; B: 21 songs, 172 pulses). C) Density map showing positions of all male acoustic centres around the females during pulse song production (23 males, 479 songs, 4338 pulses). Colors (online) indicate number of pulses produced (spatial resolution = 0.04 mm²). Outlines are to scale for average female (n=23). Grey points indicate outliers (local density below 5% maximum density).

Figure 2. (Color online) A) Distribution of male acoustic centre distances from female antennae at the time of song production. Pulse songs, 4338 pulses from 23 males (Black). Sine songs, average distance per song, 206 songs from 23 males (Red, color online). Values normalised to the total of pulse and sine songs respectively. B) Distribution of male acoustic centres around females at the time of pulse song production. Dashed line (Red, color online) depicts a 2-peak Gaussian fit to the data ($r^2=0.52$). Peaks are at 140.1° and 225.9°, half-amplitude bandwidths are 27.12° and 42° respectively. n = 4338 pulses from 23 males.

Figure 3. (Color online) A) Mean left (black line n = 5) and right (red line n = 10) antennal vibration magnitude measured using LDV. Sound stimuli are presented from different angular positions around a female *Drosophila*. Data are normalised to 92 dB_{SVL} (stimulus amplitude), Y-axis units of mm/s/mm/s. Data points are fit with a spline to allow clear presentation of the figure-of-eight pattern of antennal sensitivity. Shading indicates standard error. B) Position of male acoustic centres relative to the female antenna. Female is depicted in the centre with two males each showing unilateral wing extension behind her. The position of the outstretched male wings (at 225° and 135°) depicts the position of the two acoustic centres predominantly used by singing males. The dashed black lines indicate the trajectory of the female antenna; the grey dashed line indicates the orientation of 0°-180°. C) Mean

sensitivity (\pm standard error) measured as the transfer function ($V_{\text{vib}}/V_{\text{air}}$) between antennal mechanical response (antennal velocity, mm/s) and stimulus amplitude (particle velocity, mm/s) for the left antenna (black) and right antenna (red, color online) at the 2 angles males predominantly sing from (135° and 225°). D) Resonant frequency at different angles of sound presentation for *Oregon R* (n=9) and (E) *btv^{5P1}* (n=10) at 3 amplitudes: 88 dB_{SVL} (red), 68 dB_{SVL} (black), 48 dB_{SVL} (blue). Right antennal responses shown. Lines are spline fits to data. F) Difference between maximum and minimum response frequency, taken from the spline fit, for each fly strain (black, *Oregon R*; white, *40AG13*; grey, *btv^{5P1}*).

Figure 4. (Color online) A) Antennal resonance frequency measured using simple harmonic oscillator (SHO) fits across amplitude range. Horizontal bars: song amplitudes (median and interquartile range); vertical bars: song frequency (mean and standard deviation), dark grey shows sine songs, light grey pulse songs. B) Antennal response to changes in amplitude. SHO fits to data from a single fly shown.